1397

ACTION OF PURIFIED MILK PHOSPHATASE ON PHOSPHOSERINE AND ON CASEIN

C. A. ZITTLE AND ELIZABETH W. BINGHAM
Eastern Regional Research Laboratory,[1] Philadelphia 18, Pennsylvania

SUMMARY

The action of a purified alkaline phosphatase from milk on casein and phosphoserine has been studied. The milk phosphatase preparation has no proteolytic action on casein, but it splits inorganic phosphate from both casein and phosphoserine. The action of the milk phosphatase was studied at pH 8 to 9.5 with several concentrations of phosphoserine. The optimum action was at pH 9.5 or higher. Values for the kinetic constants K_M and $V_{max.}$ were calculated from the data. The action of the milk phosphatase on phosphoserine was similar to its action on other simple phosphate esters. The milk phosphatase was considerably less reactive with casein (about 200 times more phosphatase was required for comparable release of phosphate), and the greatest activity was at pH 6 to 7. Reasons are given for not necessarily postulating a second enzyme for this activity, but rather that it might result from the complex nature and size of the casein molecule. Both calcium-sensitive α-casein and whole casein were dephosphorylated at comparable rates. The experiments were carried to 80% dephosphorylation of the casein, and presumably complete dephosphorylation could be accomplished with this phosphatase preparation. It is suggested that some dephosphorylation of casein might occur in milk and that special precautions may be required to obtain casein preparations of maximum and constant phosphorus content.

The availability of a purified, highly active milk phosphatase (23, 26) suggested that its action on casein be studied, since a number of other phosphatases (9, 11, 17) have been found to dephosphorylate casein. At the same time, the action of the milk phosphatase on serine phosphate has been studied, since most or all of the phosphate contained in casein is probably esterified to the OH group of this amino acid (8, 9, 18) or to the amino acid threonine (3).

MATERIAL AND METHODS

Whole casein. Whole casein was prepared by acidification of skimmilk to pH 4.5 with *N* HCl. The precipitate was washed four times with water, twice dissolved and reprecipitated with acid, then dried with absolute ethanol and ether (6).

α-Casein, calcium-sensitive. A calcium-sensitive α-casein was prepared from whole casein by a modification (24) of the fractionation procedure in aqueous urea solutions (7).

Preparation of alkaline phosphatase from milk. The alkaline phosphatase was prepared from skimmilk by the method previously described (23, 26). The preparation used in the present studies contained seven units of phosphatase activity per milligram of solids. A unit of phosphatase is defined as the amount which causes the liberation of 1 μM phenol in 5 min. at 38° C. The unit of activity

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[1] Eastern Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture.

defined here conforms to a recommended practice (10) and is about 300 times larger than the unit previously used (23). The phosphatase preparation contains about 2,500 units per milligram of the previously defined units (26). The dried phosphatase preparation was suspended in water to a concentration of 0.5% for the casein experiments, 0.1% for the phosphoserine experiments. The suspension was centrifuged and the supernatant solution, which contained all of the phosphatase activity, had a protein content (nitrogen × 6.25) equivalent to 70% of the dry weight of the phosphatase preparation, and the specific activity is about ten units per milligram of protein in solution.

Assay of alkaline phosphatase. The assays were run at 38° C. in an ethanolamine-HCl buffer of pH 9.8 with phenylphosphate as the substrate (25). The liberated phenol was determined colorimetrically (25) in the Beckman Model B spectrophotometer at a wave length of 650 mμ. The phenol equivalence was read from a standard curve relating phenol and color.

Action of the phosphatase on phosphoserine. The action of the phosphatase was determined by measuring the inorganic phosphate released from phosphoserine (commercial source) in 30 min. at 38° C. Two milliliters of the incubation mixture contained 1.6 ml. veronal-acetate buffer (12) in the pH range 8.0 to 9.5, 6 μM $MgCl_2$, 10 γ milk phosphatase, and 0.0125 to 5 μM phosphoserine. The reaction was stopped by the addition of 0.5 ml. of 0.38 N H_2SO_4. The inorganic phosphorus released was determined by the method of Dryer *et al.* (4). The optical density was measured in the Beckman DU spectrophotometer at 345 mμ and the phosphate estimated from a standard curve relating optical density and phosphate.

The action of phosphatase on caseins. The caseins, either whole casein or the calcium-sensitive α-casein, were dissolved using 0.1 N NaOH to obtain the approximate pH desired. Veronal-acetate buffer (12) of the desired pH was added and the pH was readjusted, if necessary, with 0.1 N NaOH or HCl. Five milliliters of incubation mixture contained 1.25 ml. buffer, 15 μM $MgCl_2$, 25 mg. casein, except where stated otherwise, and usually 5 mg. of the milk phosphatase. The solutions were incubated for various times at 38° C. The reaction was stopped by adding 1 ml. incubation mixture to 1 ml. 0.02 M silicotungstic acid, then 2 ml. of 20% trichloroacetic acid was added and the mixture centrifuged. The inorganic phosphate was determined on 2 ml. of the supernatant solution by the method of Sumner (21). The optical density of the developed color was measured in the Beckman DU spectrophotometer at 650 mμ.

RESULTS

The conditions for the optimum interaction of the alkaline phosphatase of milk with the substrate phosphoserine were investigated, with the thought that the results might suggest the best conditions for the interaction of alkaline phosphatase with casein. The phosphate of casein is probably bound through the hydroxyl groups of the amino acids serine or threonine, since phosphopeptides obtained from casein have the phosphate bound to the serine (8, 9, 18) or threo-

nine (3). The action of the phosphatase on phosphoserine was studied at several pH values, to determine the optimum pH, and also with several concentrations of the substrate, phosphoserine, to determine the enzyme-substrate dissociation constant, K_M, as a measure of the affinity between substrate and enzyme. The latter also provided data for estimating the concentration of substrate, giving the maximum velocity, $V_{max.}$, for the reaction. At a concentration of phosphoserine suitable for assay purposes, namely 125×10^{-5} M per liter, the pH optimum had not been reached even at pH 9.6. As with other alkaline phosphatase reactions, the pH optimum is a function of substrate concentration and with 25×10^{-5} M phosphoserine per liter, the optimum is at pH 9.0.

The K_M and $V_{max.}$ for the action of the milk phosphatase on phosphoserine were computed from the integrated form of the Michaelis-Menten equation developed by Walker and Schmidt (22).

$$(S_o - S_t) \cdot t = -K_M (2.3 \log S_o/S_t) \cdot t + V_{max.}$$

S_o is the initial substrate concentration, and S_t is the substrate concentration after the action of the enzyme for time t. By a plot of $(S_o - S_t) \cdot t$ versus $(2.3 \log S_o/S_t) \cdot t$, a straight line is obtained. The slope is $-K_M$ and the intercept on the $(S_o - S_t) \cdot t$ axis is $V_{max.}$ The use of this equation made it possible to include data at low substrate concentrations for the calculation of K_M and $V_{max.}$, a substrate region where reaction rates are linear for only a brief time. The results are presented in Figure 1, where pK_M $(= 1/\log K_M)$ is plotted against pH, and in Figure 2, where $V_{max.}$ is plotted against pH. The large pK_M value at pH 8 means that the substrate concentration has to be less than 2.5×10^{-5} M

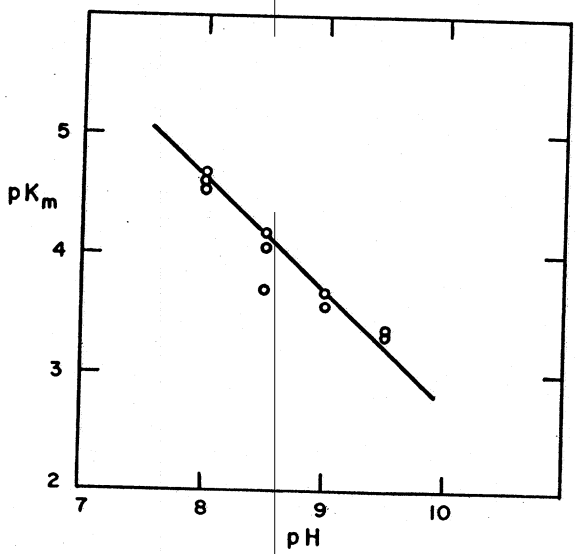

FIG. 1. The enzyme-substrate dissociation constant $(pK_M = \log 1/K_M)$ for the action of the milk alkaline phosphatase on the substrate phosphoserine at various pH values. K_M is expressed in moles per liter.

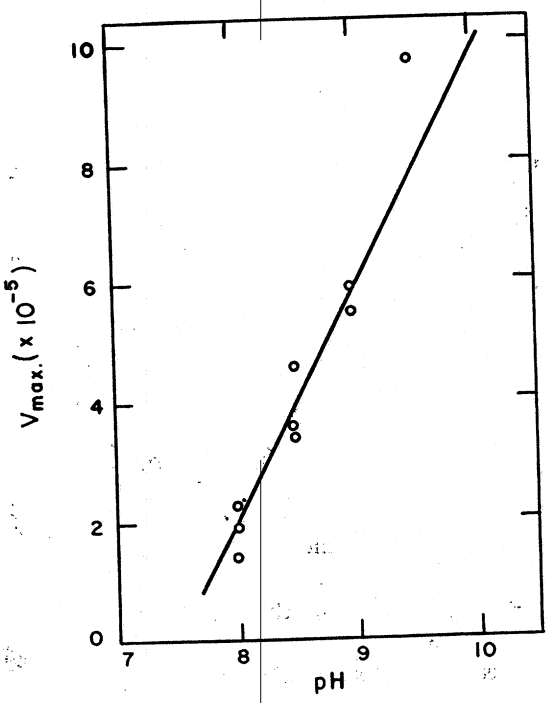

FIG. 2. The maximum velocity, $V_{max.}$, for the action of the milk phosphatase on phosphoserine at various pH values. $V_{max.}$ is expressed in moles per liter per 5 min.

per liter before the reaction becomes dependent on substrate concentration. This made it impractical to estimate the K_M values at pH values below eight, since these concentrations of inorganic phosphate released were too small to be estimated accurately.

The results with phosphoserine, although of general interest, did not serve as a guide for the action of the milk phosphatase on casein. The milk phosphatase preparation did dephosphorylate casein, but much larger concentrations (about 200 times) of phosphatase were required than for phosphoserine, and dephosphorylation of casein was more rapid at pH 6 than at pH 10. A detailed comparison of the action of the milk phosphatase on the two substrates will be discussed later.

Both whole casein and calcium-sensitive α-casein were dephosphorylated to about the same extent. The results with the two caseins and several concentrations of the milk phosphatase are shown in Figure 3. The reason for the nonlinear release of phosphorus as the amount of phosphatase is increased is not clear. It was not due to a decrease in the phosphatase activity, for all of the activity (tested with the substrate phenylphosphate) was still present at the end of the experiment. In fact, the casein stabilized the phosphatase, for phosphatase stored at this pH and temperature for the same length of time with no casein present had lost some activity. The ninhydrin reaction (13) applied to this

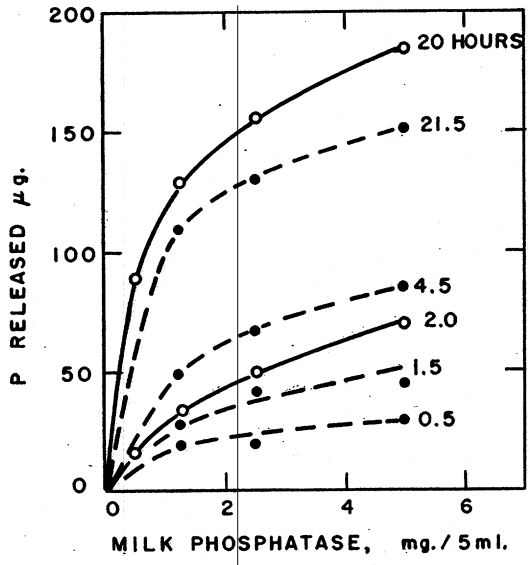

FIG. 3. Action of milk prosphatase on whole casein (pH 7.0) and calcium-sensitive α-casein (pH 6.4) for various periods of time, measured by the release of inorganic phosphate. ●——● Whole casein (177 γ casein-P in 5 ml.); ○——○ α-casein, calcium-sensitive (250 γ casein-P in 5 ml.). Numbers at ends of curves indicate hours that the phosphatase acted.

mixture remained the same after 0, 2, and 20 hr.; hence, the milk phosphatase preparation contained no proteolytic activity for the conditions used.

The data in Figure 4 show the influence of time and pH on the dephosphorylation of calcium-sensitive α-casein by the milk phosphatase. The maximum rate of hydrolysis results at pH 6 and 7. The shape of these curves—rapid initial hydrolysis followed by a considerably slower rate—is characteristic of this dephosphorylation.

The effect of the concentration of casein on the dephosphorylation activity of the milk phosphatase is shown in Figure 5 for pH 6.5 and 9.0. K_M and $V_{max.}$ values were calculated by the procedure described earlier for phosphoserine. Straight lines were obtained when $(S_o - S_t) \cdot t$ was plotted against (2.3 log $S_o/S_t \cdot t$; hence, for these conditions, the catalytic activity of the phosphatase appears to represent the usual summation of zero- and first-order rates.

DISCUSSION

Chemical properties of phosphoserine have been described (16) and a few reports of the action of phosphatases on this compound have appeared. A preparation from calf intestinal mucosa will dephosphorylate phosphoserine as rapidly as it does glycerophosphate (19), presumably due to the alkaline phosphatase which is present in intestinal mucosa in large amounts. Phosphatases also have been reported (15, 20) that are specific for phosphoserine, acting only on this substrate. They will also phosphorylate serine.

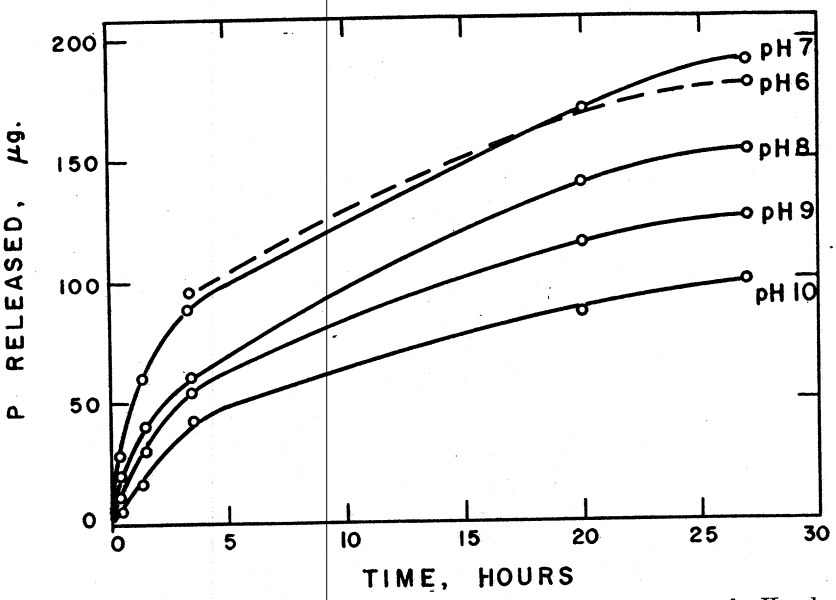

FIG. 4. Action of milk phosphatase on calcium-sensitive α-casein at several pH values, for various periods of time, measured by the release of inorganic phosphorus. The amount of milk phosphatase in this experiment was 2.5 mg. in the 5-ml. reaction mixture.

The present studies show that the action of the milk alkaline phosphatase on phosphoserine is very similar to its action on other simple phosphate esters. Phosphoserine and phenylphosphate are hydrolyzed equally rapidly by the milk phosphatase (23); with phosphoserine, however, the phosphatase has the same

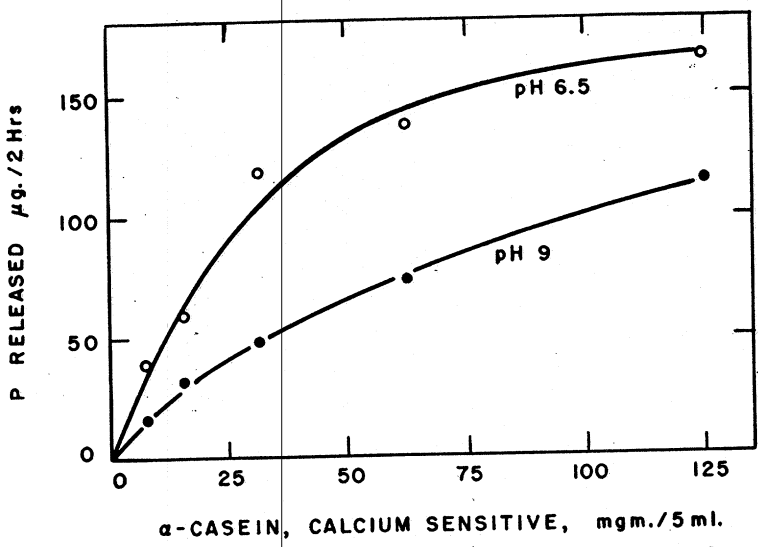

FIG. 5. Action of milk phosphatase on calcium-sensitive α-casein with several concentrations of the casein at pH 6.5 and 9.0, measured by the release of inorganic phosphorus in 2 hr.

pK_M-pH values as with *o*-carboxyphenylphosphate (23). The milk phosphatase, acting on phosphoserine, also has its greatest activity at pH 9.5 or above. The milk phosphatase preparation used in the present study contained no acid phosphatase, that is, a phosphatase active at pH 4, which has been reported in milk (14). Large amounts of the milk phosphatase were incubated at 35° C. at pH 4.0 with phenylphosphate and with phosphoserine with negative results.

The information about the action of the milk phosphatase on phosphoserine provided no guide to its action on casein. The milk phosphatase does dephosphorylate casein, but large amounts of the phosphatase are required, and the most marked difference is that dephosphorylation is more rapid at pH 6 to 7 than at pH 9.5. As noted above, the phosphatase preparations did not contain an acid phosphatase, but the above pH data suggest that a second dephosphorylating enzyme might be present. Previous studies (26) had showed that the purified milk phosphatase preparations contained an enzyme-hydrolyzing nucleic acid, and there well might be other phosphorylitic enzymes in the milk phosphatase preparations also. The pH data, however, do not necessarily require that the enzyme-dephosphorylating casein be different from the enzyme-hydrolyzing phosphoserine and other simple phosphate substrates. The casein is a large molecule and there might be a local charge on its molecule at the higher pH values that would hamper enzyme-substrate union and consequent dephosphorylation of the casein.

At pH 6, on the other hand, the unfavorable charge might be absent, thus permitting action of the phosphatase, although at a low level. A phosphatase preparation from calf intestinal mucosa will dephosphorylate casein (11) but here, too, although it is a potent source of alkaline phosphatase, the greatest activity on casein is at pH 7.4. A kinetic analysis of the action of the milk phosphatase on casein (Figure 5) shows that this action has a greater affinity between enzyme and substrate (smaller K_M value) at pH 6.5 than at pH 9.0. In this respect, the effect of pH is similar to the effect on the action of the phosphatase on phosphoserine. The actual values for K_M are 1.5 mM casein-P per liter (0.5% casein) at pH 6.5, and 4.5 (1.5%) at pH 9.0. These values are of the same magnitude as others have reported for enzymatic dephosphorylation of casein. The value for the spleen phosphatase acting on casein at pH 5.5 was 3 mM casein-P per liter (9).

Calcium-sensitive α-casein was chosen for the present studies to have a casein that was free of other interacting components. This choice was motivated by the report (17) that whole casein was very poorly dephosphorylated by phosphatase, whereas the separated α- and β-caseins were readily dephosphorylated. Recent studies (9, 11), however, have not confirmed this claim, and the present studies show that the calcium-sensitive α-casein and whole casein were dephosphorylated at comparable rates.

The results of the present study raise the question whether the action of the phosphatase in milk might lead to caseins of variable phosphate content, depending on the time of isolation after milking and other factors. The amount of phosphatase used in the present studies, although large, to obtain a readily measur-

able dephosphorylation, is only about ten times greater than the concentration found in milk. There is about 0.7 unit (defined above) of phosphatase per milliliter of milk (0.7 unit to about 30 mg. casein); whereas, the purified phosphatase contained seven units per milligram and, as shown in Figure 3, 1 mg. of this phosphatase gives a marked dephosphorylation of 25 mg. of casein.

It is well known that the inorganic phosphate in milk increases on standing, but analysis indicates that all of this increase seems to be accounted for by a decrease in the soluble, low molecular weight phosphate esters in milk (5). The relatively large contribution of this fraction to an increase in the inorganic phosphate might, however, mask a contribution from dephosphorylation of casein. Changes in the phosphatase content of cow's milk by the administration of thyroxine or thiouracil bring about changes in the phosphorus content of the casein (1). Thyroxine decreases the phosphatase in milk and the phosphate content of the casein goes up; thiouracil increases the phosphatase content of milk (as much as threefold above normal) and the phosphate content of casein goes down. A somewhat similar pattern emerged from changes in the phosphatase during the course of lactation (2). The authors (1) attributed the changes in the phosphate content of casein to metabolic changes within the mammary gland. The present studies, however, suggest that the change in the phosphate content of casein may have taken place after the milk was formed. Also, it appears that special precautions will be required to obtain casein preparations of maximum and constant phosphate content.

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